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14. ABSTRACT

Trauma-hemorrhagic shock (T?HS) is a major consequence of battlefield injury as well as civilian trauma and one of the major causes of death in these patients is the multiple organ dysfunction syndrome or MODS. Although the exact cause of MODS is incompletely understood, it appears related to normal body responses getting out of control resulting in the excessive activation of this research is to better understand the triggers that cause MODS and to test new therapies directed at controlling these over active and hence tissue-inurious pathways. Two major significant observations were made during the funding period. 1) These results validated the hypothesis that the biologic factors that start the process leading to MODS originated from the stressed intestine and/or its contents and that these factors reached the systemic circulation via the intestinal lymphatic system. 2) Secondly,we documented that the drug C1inh given after the shock-trauma insult, as part of the volume resuscitation regimen, limited to some extent T/HS-induced MODS.

15. SUBJECT TERMS- Trauma-shock, MODS, Acute lung injury, gut-lymph, Complement 1 inhibitor, C1inh

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INTRODUCTION: Trauma hemorrhagic shock is a major consequence of battlefield injury as well as civilian trauma. In battlefield casualties, where it is difficult to have large amounts of resuscitative fluids in far forward positions, it is critical to develop strategies by which the secondary effects of shock, including multiple organ failure, can be ameliorated or prevented. We have results indicating that trauma-hemorrhagic shock (T/HS)-induced early multiple organ dysfunction (MODS) syndrome is related to gut injury and lipid and protein factors exiting the stressed gut via the intestinal lymphatics. Consequently, the goal of this project will be to perform basic research on the role of gut-origin MODS after T/HS and to study two potential mechanism-based therapies for preventing or limiting the development of multiple organ failure after traumatic shock. FYT720 (sphingosine-1 phosphate agonist), was chosen as one therapy and will take advantage of the unique ability of FYT720 to decrease the activities of the innate and adaptive immune systems while maintaining endothelial cell barrier function and vascular homeostasis during stress states. Hence FYT720 has the ability to limit tissue damage and excessive systemic inflammation thereby reducing the development or magnitude of trauma-induced MODS. Complement (C) inhibition was chosen as the second therapy, since C activation has been implicated in ischemia-reperfusion-mediated organ injury and is increased early after trauma in patients with its level of activation being proportional to injury. Thus, the 3 basic hypotheses of this proposal are: (1) Gut derived factors in lymph are responsible for acute trauma hemorrhagic shock induced MODS; (2) That FYT720 through its immuno-inflammatory and vascular effects will limit T/HS-induced inflammation and MODS. (3) That systemic complement activation is a key factor in T/HS-induced gut injury and the production of tissue injurious factors in T/HS lymph and consequently inhibition of complement activity would limit gut injury and protect against T/HS-induced lung injury and MODS. Importantly, these hypotheses are being tested initially in a rodent model and if the therapy appears beneficial, it will be validated in a pig model. This escalation of species from rat to pig is critical in validating potential clinical utility since positive results in a pig model are more likely to accurately predict positive results in patients. It is also important to stress that both FYT720 and the C1 inhibitor are approved for human use in other conditions thereby facilitating clinical trials if drugs give promising results.

BODY: Overview: The proposal's time line was to complete AIM 3 in the first 4 quarters of the funding period (4/12/12 through 4/11/13) and complete AIM 1in the second 4 quarters (4/12/13 through 4/15/14). The primary goal of Aim 3 was to test the global hypothesis that the sphingosine 1phosphate (S1P) agonist, FYT720, would prevent/limit trauma-hemorrhagic shock-induced multiple organ dysfunction syndrome when administered using a post-treatment strategy. We have completed the majority of the work proposed in AIM 3 and have finalized a CRADA/MTA with Dr JJ Dallelucce to carry out studies measuring complement activity on the collected intestinal lymph and plasma samples. During the current funding period, the strategy was to inhibit complement activation with a human recombinant C1-inhibitor (C1-INH) obtained from Behring Laboratories, since complement activation has been implicated in the pathogenesis of T/HS and other ischemia-reperfusion models of gut injury as well as in the pathogenesis of trauma- and stress-induced lung injury and SIRS. Specifically, complement has been shown to be activated early after trauma and injury with its level of activation being directly proportional to the severity of the injury. Furthermore, complement activation is an important component in the inflammatory cascade that leads to tissue damage and organ dysfunction in the early phase of trauma.

To date, progress is well aligned with the schedule and with the exception of running assays on collected specimens, all of the proposed work has been completed. Also, during the second year of funding, we completed the blood flow studies on the effect of FYT720 on organ blood flow in addition to the C1inh work.

Section I. Experiment 1: *Test the hypothesis that trauma-hemorrhagic shock (T/HS)-induced MODS is prevented by the administration of C1inh as part of a fluid resuscitation regimen.*

The first major set of key observations from this experiment was that post-shock treatment with C1inh at a dose of 200 units/kg abrogates acute lung injury but is less effective at limiting T/HS-induced neutrophil priming or red blood cell dysfunction (Table 1). Thus, the major beneficial effect of the administration of FYT70 given during the post-T/HS resuscitation period was to limit T/HS-induced increases in lung permeability (acute lung injury).

Table 1: Effect of C1inh (200 Units/kg) on T/HS-induced responses

Group	Lung-% EB In BALF	Post-PMN RB	Post-RBC CD36	Post-RBC EI
T/SS	1.6 ± .4	234 ± 13	0.4 ± .2	6.2 ± .2
T/SS-C1inh	1.6 ± .2	237 ± 11	0.9 ± 1.3	6.3 ± .2
T/HS	2.9 ± .4 *	346 ± 37 *	15 ± 4 +	4.6 ± .1 +
T/HS-C1inh	1.6 ± .4	315 ± 38 #	12.5 ± 4 +	5.2 ± .9

Data expressed as Mean ± SD with n=6 rats/group. * $p < 0.01$ vs all other groups. # $p < 0.01$ vs T/SS groups at that time point; + $p < 0.05$ vs T/SS group at that time point

→Lung injury (permeability) measured by the amount of evan's blue (EB) dye in bronchoalveolar fluid (BALF). The data is expressed as a percentage of the level of EB in the lung BALF divided by the level of EB in the blood after a systemic injection of EB.

→ PMN priming was measured by flow cytometry after stimulation with PMA. Thus, respiratory burst (RB) activity is expressed as units. Post-PMN samples were collected at 3 hrs after the end of the T/SS or T/HS shock period.

→ RBC CD36 is expressed as the percentage of RBCs expressing surface CD36.

→ RBC dysfunction was assessed by measuring RBC deformability (EI). EI reflects the length that RBCs can be elongated with a constant shear stress. Thus, a lower EI reflects a loss of RBC deformability. Post-RBC samples collected at 3 hrs after the end of the T/SS or T/HS shock period. Data expressed at 10^{-2} Units.

Having shown that C1ih, at a dose of 200 Units/kg was effective (Table 1), a dose-response study of C1inh was performed to see if the lung protective effect of C1inh persisted with lower doses. The results of this study showed that the beneficial effects of C1inh at lower doses was largely lost (Table 2). However, at a dose of 100 units/kg C1inh did limit T/HS-induced PMN priming and RBC rigidity. A full dose response study was also done with T/SS rats but since these values were not different from each other or the T/SS vehicle shown in Table 1, for clarity, this data will not be shown.

Table 2; Effect of multiple doses of C1inh

Group	Lung - %EB BALF	Post - PMN RB	Post - RBC CD36	Post-RBC EI
T/HS-vehicle	2.9 ± .4	346 ± 37	15.4 ± 4	4.6 ± .1
T/HS – 25 Units	2.5 ± .6	289 ± 25	7.8 ± 7	5.4 ± .1
T/HS – 50 Units	2.8 ± .4	287 ± 105	7.3 ± 7	5.9 ± .9
T/HS – 100 Units	2.8 ± .8	248 ± 24 +	10.1 ± 10	6.2 ± .7 +
T/HS – 200 Units	1.6 ± .4 *	315 ± 38	12.5 ± 4	5.2 ± .9

Data expressed as Mean ± SD with n=6 rats/group.
*p < 0.01 vs all other groups
+ p <0.05 vs T/HS vehicle (similar to T/SS control group; data not shown)

In summary, the C1inh dose study indicated that lung protection was greatest with the 200 Unit/kg dose, while the most effective dose to limit PMN priming and RBC rigidity was 100 Units/kg.

Other variables measured included the volume of blood required to be withdrawn to induce and maintain the shock state,to show that the groups were physiologically similar. There were no differences between the T/HS animals receiving vehicle and the T/HS groups receiving different doses of C1inh (Table3).

Table 3: Effect of FYT720 on blood removed

Group	Blood withdrawn (ml/kg)
T/HS + Vehicle	26 ± 3 ml/kg
T/HS + 25 Units	30 ± 3 ml/kg
T/HS + 50 Units	27 ± 2 ml/kg
T/HS + 100 Units	28 ± 3 ml/kg
T/HS + 200 Units	27 ± 2 ml/kg

Data expressed as Mean ± SD with 6 rats per group.

Other data collected including hemodynamic response and hematologic data (WBC count, lymphocyte count, platelet count and Hematocrit) showed that T/HS groups receiving vehicle or C1inh were similar (data not shown).

Lastly, plasma and serum samples have been collected and stored in order to measure complement activity using the broad panel of assays proposed in this grant. These stored samples will be sent to Dr JJ DalleLucce with samples collected from Experiment 2 described below.

Conclusion: The results of this project so far indicate that the S1P agonist, FYT720 (Aim 3 reported in year 1) is more protective that C1inh in a rodent post-therapy T/HS model. This based on fact that C1inh was less able to limit lung injury, neutrophil priming and RBC dysfunction than FTY720 (FYT 720 data in year 1 annual report).

Section II. Experiment 2: *Test the hypothesis that one of the major mechanisms by which C1inh prevents/limits T/HS-MODS is by a protective effect on the gut and/or on the biologic activity or lymphocyte count of T/HS mesenteric lymph.*

This hypothesis is based on studies showing that gut injury and the generation of biologically active factors in intestinal lymph are responsible for the early signs of acute lung injury and systemic inflammation after T/HS. To accomplish this goal, we quantified gut injury and will measure the biologic activity of T/HS intestinal lymph. Gut injury was quantified by measuring changes in gut permeability in one group of animals, while a second group had lymph collected from their mesenteric lymph ducts. These are termed lymph duct cannulated (LDC) rats. To assess the biologic activity of mesenteric lymph, the rats had a catheter placed into their main lymphatic duct draining the intestine to collect lymph samples for subsequent analysis. T/SS rats receiving vehicle or C1inh served as controls. In vitro studies measuring the biologic activity of mesenteric (intestinal) lymph samples from rats subjected to trauma-hemorrhagic shock (T/HS) or trauma-sham shock (T/SS) receiving vehicle or C1inh were carried out. In these in vitro studies normal whole blood will be incubated with lymph (10% v/v) from one of these groups of rats and the effects of these lymph samples on neutrophil activity (respiratory burst) and RBC function (deformability and adhesion to endothelium) will be measured. These in vitro studies are in progress.

To assess the potential protective effect of C1inh on T/HS-induced gut injury, we measured gut permeability using the permeability probe FD4, which is a fluorescent 4kD dextran molecule. In this assay, FD4 is placed in the lumen of the gut and 30 minutes later plasma samples are collected and the blood FD4 concentration is measured. Blood FD4 levels thus represent the amount of FD4 that has crossed the intestinal barrier. A series of doses of C1inh were tested than ranged from 25 to 200 Units/kg (Table 4).

We found that, all of the doses of C1inh tested reduced gut permeability in rats subjected to T/HS as compared to T/HS vehicle-treated animals (T/HS + Vehicle vs all T/HS+C1inh; $p < 0.01$). However as also shown in Table 4, only the 200 Unit dose of C1inh totally presented the T/HS-induced increased gut permeability. At all of the lower doses, the C1inh was only partially protective.

Table 4; Effect of C1inh on T/HS-induced gut permeability

C1inh dose	T/SS animals	T/HS animals	p value vs T/SS
Vehicle	0.34 ± .18	1.52 ± .64 *	< 0.01
25 Units	0.28 ± .14	0.72 ± .38	< 0.05
50 Units	0.26 ± .14	0.54 ± .13	< 0.01
100 Units	0.36 ± .12	0.58 ± .20	< 0.05
200 Units	0.37 ± .09	0.50 ± .13	0.12

All data expressed as Mean ± SD with n = 6 rats /group

* $p < 0.01$ vs all C1inh-treated groups

Morphologic studies of the intestinal villi are also planned to complement the physiologic FD4 studies with morphologic studies. Gut samples have been all harvested and final processing is in progress.

Effect of mesenteric lymph duct cannulation with or without C1inh on T/HS-induced organ injury and on RBC or PMN function.

Acute lung injury, gut injury, PMN priming and RBC function was also measured in addition to collecting lymph, plasma and tissues samples for complement assays in these groups of animals that had their intestinal lymph collected. These measurements were made even though our previous work documented that removing gut lymph prevented these changes. The rationale for doing this work is two fold. First, the results would be important in validating previous studies showing that T/HS-induced lung injury, PMN priming and RBC injury could be abrogated by preventing intestinal lymph from reaching the systemic circulation (this was accomplished by lymph duct catheters). Secondly, these results would help determine the relative effectiveness of C1inh in combination with intestinal lymph diversion as a protective strategy after T/HS.

We first measured gut permeability in the lymph duct ligated rats using FD4. Two doses of C1inh were used; a 200 Unit dose since it was shown to be gut protective (see Table 4 above) and the 100 Unit dose since this dose was most effective in limiting PMN priming and RBC rigidity (Table 2 above). We found that the 200 Unit but not the 100Unit dose of C1inh was able to prevent the T/HS-induced increase in gut permeability in these LDC (Table 5).

Table 5; Effect of C1inh in limiting T/HS-induced increases in gut permeability in lymph duct diverted (LDC)rats.

Group	T/SS	T/HS	P value T/SS vs T/HS
Vehicle	0.34 ± .07	0.65 ± .20	< 0.05
100 Units C1inh	0.31 ± .12	0.54 ± .12	< 0.05
200 Units C1inh	0.37 ± .13	0.47 ± .17	Not significant
ANOVA	0.63	0.34	

Data expressed as mean ± SD with n=6-8 animals group
Anova is used to compare the 3 T/SS and 3 T/HS groups to each other.

As predicted from the earlier lymph diversion studies, both the T/HS-vehicle and T/HS-C1inh LDC groups did not manifest acute lung injury when lymph was prevented from entering the systemic circulation (Data not shown).

However, the addition of the C1inh was additive to LDC in terms of limiting T/HS-induced PMN priming and upregulation of RBC CD36 expression (Table 6). However, no differences in RBC deformability (EI) were found the groups.

Table 6: Effect of C1inh on RBC and PMN function in LDC rats

Group	PMN RB T/SS	PMN RB T/HS	t-test	RBC % CD36 T/SS	RBC % CD36 T/HS	t-test
Vehicle	237 ± 13	330 ± 38 *	< 0.001	0.4 ± 0.3	15.4 ± 4.0 *	< 0.001
C1inh 100U	241 ± 10	263 ± 39	0.10	1.3 ± 1.2	5.0 ± 3.4	0.03
C1inh 200H	233 ± 12	274 ± 29	0.06	1.6 ± 1.5	9.0 ± 5.0	< 0.01
ANOVA	0.52	< 0.01		0.20	< 0.01	

Data expressed as mean ± SD with n=6-8 animals group
Anova is used to compare the 3 T/SS and 3 T/HS groups to each other.
* $p < 0.05$ vs C1inh T/HS groups

Additionally, this work provides additional controls for the plasma and serum complement studies carried out in Experiment 1. That is, the effect of T/HS and C1inh on systemic complement activity can be determined both in the presence and absence of the systemic effects of T/HS lymph and thereby allow the systemic protective effects of C1inh to be compared with the protective effects of lymph diversion.

RESEARCH ACCOMPLISHMENTS In year 2:

1) C1inh administered as part of the fluid resuscitation therapy is able to abrogate to some extent T/HS-induced acute lung injury, neutrophil (PMN) priming and RBC injury (Tables 1 & 2).

These results are encouraging since they indicate that post-injury therapy with some of the doses of the C1inh tested are able to reduce lung injury and neutrophil priming, which are two of the major consequences of trauma-hemorrhage that have been associated with increased morbidity and mortality.

2) The protective effect of C1inh on T/HS-induced acute lung injury, systemic inflammation and RBC dysfunction may be related, at least in part, by C1inh's ability to limit gut injury and dysfunction (Tables 4). To validate this notion, we have collected mesenteric lymph samples from vehicle and C1inh-treated rats to assess its bioactivity.

If C1inh limits mesenteric lymph bioactivity, these results would support the gut lymph hypothesis of early acute MODS after T/HS and would be consistent with our previous work in this field as well as the work of others. Furthermore, it supports the underlying concept that factors liberated into the systemic circulation by the stressed gut significantly contribute to the transduction of the hemodynamic shock state to a systemic inflammatory state.

3) As predicted, diverting intestinal lymph from reaching the systemic circulation is sufficient by itself to prevent T/HS-induced acute lung injury and neutrophil priming (Table 6).

These results support the gut lymph hypothesis of MODS and further support the potential importance of C1inh's ability to limit the gut injury.

4) Taken together, these rodent studies have validated the therapeutic potential of C1inh to limit T/HS-induced acute gut injury, lung injury, neutrophil priming and RBC injury. However, the potential clinical value of C1inh is limited by the observation that only certain of the doses tested had biologic effects and the biologic effects on specific cells and organs varied by dose.

REPORTABLE OUTCOMES:

Publications:

Bonitz, JA, Son JY, Chandler B, Tomaio JN, Qin Y, Prescott LM, Feketeova E, Deitch EA. A Sphingosine-1 Phosphate agonist (FTY720) limits trauma/hemorrhagic shock-induced multiple organ dysfunction syndrome. Shock (in press)

Presentations: (Need to update)

Bonitz JA, Chandler B, son J, Deitch EA. FYT720 effect on trauma/hemorrhagic shock-induced gut injury. To be presented at 36th annual meeting of Shock Society, San Diego CA. June 3rd, 2013.

CONCLUSIONS:

The major findings of this work validate the goals for which the grant was funded. Specifically, to test potential drugs that could be effective in limiting the later complications observed after trauma-hemorrhage, especially the development of acute lung injury and MODS. Although the results are very encouraging, it appears that FTY720 may have more clinical potential than C1inh. Additionally, since the FTY720 and C1inh studies were carried out in a rodent model, the ability to directly apply these results to patients may be limited. Hence, one of the next steps proposed in the grant is to test if the positive FTY720 rodent studies could be validated in a pig model. These studies have been initiated.

PROPOSED STUDIES FOR YEAR 3:

In year 3 (final year of the grant), we propose to accomplish the following 4 objectives:

Objective 1: Complete the in vitro studies testing where administration of C1inh to rats subjected to T/HS abrogates the biologic activity of mesenteric (intestinal) lymph. All of the lymph samples have been collected. In this set of studies, the ability of mesenteric lymph to prime neutrophils and/or cause RBC dysfunction will be determined. Naïve rats will be the blood donors.

Objective 2: Continue collaboration with Dr JJ DalleLucce to assess Complement activation after T/HS and the modulating effects of therapy with FTY720 as well as the C1inh. All of the FTY720 specimens have been shipped to Dr JJ DalleLucce and we are waiting for the results. All the C1inh samples have been collected and will be shipped to Dr JJ DalleLucce when the FTY720 assays are completed. Based upon the results, if more samples are needed, we will do additional animals.

Objective 3: Complete porcine studies testing FTY720 as described in the funded proposal (Aim 4 of proposal). We do not believe it is worthwhile to carry out any pig C1inh studies, since the rodent studies were not as promising as the FTY 720 studies.

Objective 4: Carry out proposed studies where mesenteric lymph from T/HS-treated animals receiving FTY720 or vehicle is injected into naïve mice. The goal of this study is to assess the in vivo activity of gut lymph versus its in vitro activity.